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Biomarker Response in Sheim (*Acanthopagrus latus*) Exposed to Polycyclic Aromatic Hydrocarbons

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Marine pollution is one of the major concern of oil producing states surrounding the Arabian Gulf because of their dependence on this semi-enclosed highly stressed water body. The Gulf receives 2 to 4 million barrels of crude oil each year. Over 30% of the world's oil tankers cross the waters of the Gulf to load crude oil from the 25 major oil terminals located in the coastal areas. Following the 1991 Gulf War, concern was expressed over the polycyclic aromatic hydrocarbons (PAHs) and heavy metal concentrations arising from the massive oil spillage and the oil-field fires (Al-Yakoob et al., 1993; Fowler et al., 1993). Because of the high efficiency of PAH accumulation by aquatic organisms from water (Stehly et al., 1990; Hellou et al., 1994), waterborne exposure to PAHs poses a serious toxic threat to these organisms. These groups of compounds include highly toxic and carcinogenic compounds. Studies conducted on an area polluted with creosote (a mixture of petroleum products) revealed higher incidence of neoplasm in the fish population (Malins et al., 1985, 1990; Kocan, et al., 1985). BaP, a PAH, is also present in petroleum products and in industrial waste material, and has both mutagenic and carcinogenic properties.

Traditionally, pollution levels and types of specific compounds present in the water bodies are chemically determined. It is becoming increasingly difficult and expensive to monitor all of the chemicals that comprise a complex material like crude oil. Another approach involves the determination of biological effects, although the ultimate arbiter of environmental impact, is slow to yield results. Efforts are going on for further sophistication in biomonitoring and attention is focused on specific biochemical response in an organism that can be used as a biomarker (an early warning) of environmental chemical exposure.

The cytochrome P450 1A, chosen as the biomarker for this study, is considered to be an early warning and defensive system (Beyer and Goksøyr, 1993; Arinc and Sen, 1994; Williams et al., 1997; Whyte et al., 2000). Specific cytochrome P450 isozyme 1A1 (ethoxyresorufin-o-deethylase, EROD isozyme) is induced by planar organic compounds including PAHs (Goksøyr, 1991; Goksøyr et al.,1991a,b; Goksøyr and Forlin, 1992, Raza et al., 1995). Currently, there are several ways in in which the induction of cytochrome P450 1A genes can be detected. These

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include measurement of cytochrome P450 1A catalytic activity, measurement of cytochrome P450 1A protein by immunochemical methods, or by measurement of cytochrome P450 1A mRNA content. The measurement of the catalytic activity of induced protein is the approach most readily available to the largest number of investigators. EROD is catalysed primarily by cytochrome P4501A1 in fish liver, and 7-ethoxyresorufin is now more commonly employed as the substrate because it is not hazardous, and a direct kinetic assay based on the distinct fluorescence characteristics of the product resorufin is available. Therefore, in our study a relationship was experimentally determined between PAHs exposure and biomarker response in sheim (*Acanthopagrus latus*) a native fish of the Arabian Gulf. The dose-response relationship will be useful in biomonitoring of PAHs pollution in marine environment.

MATERIALS AND METHODS

Sheim were cultured at the Mariculture and Fisheries Department (MFD) of Kuwait Institue for Scientific Research (KISR), for the study. The stock culture was raised right from the hatching of eggs. Eggs were collected from the brood stock and after fertilization they were kept in seawater in large fiberglass tanks. The seawater was pumped in from the coastal area and subjected to sand filtration. On hatching, live phytoplankton cultures, zooplankton-like rotifers and artemia larvae were fed to fry. Subsequently, commercial feed (INVE Aquaculture, Belgium) in powder and pellet form was given. feed brands such as of Aqualine, Biomar and Ecoline are procured from. Adult fish samples were provided from the stock culture for the experiment. After collection, the fish were held in filtered seawater at 22°C for at least three days to acclimatize them to laboratory conditions. A representative sample of the fish were exposed to BaP.

BaP was dissolved in dimethyl sulfoxide (DMSO) to prepare a stock solution, which was then mixed at different concentrations with corn oil. The corn oil, containing the desired concentrations of BaP was mixed with fish food and given orally to each fish. After exposure, the fish were held for four days and sacrificed as quickly as possible by blows to the head. Tissues were dissected carefully, avoiding rupturing the gall bladder (bile may contain monooxygenase inhibitors). The tissue was removed immediately and transferred into a beaker on ice. Excess blood was removed from the tissue, blotted with filter paper and weighed. The samples were either processed fresh or stored in a freezer at -80°C.

The method for the preparation of liver microsomes and assay of EROD was according to Beyer and Goksoyer (1993). Liver tissue was homogenized in 0.1M phosphate buffer, pH 7.4, containing 0.15M KCl, 1mM EDTA and 1mM DTT using Potter-Elvehjem homogenizer. The homogenate was centrifuged at 12,000 x g for 20 min at 4 °C and the supernatant (post mitochondrial) was subjected to centrifugation at 100,000 x g for 1 h in Sorvall ultracentrifuge. The pellet (microsomes) thus obtained was suspended in homogenizing buffer additionally containing 20% glycerol. The microsomes suspension was either analyzed

immediately or stored at -80 °C in small fractions for later analysis. EROD (7-ethoxyresorufin-o-deethylase) was assayed by following the conversion of ethoxy resorufin to resorufin spectroflurometrically at 535 nm and 585 nm excitation emission wavelengths. The reaction mixture contained 1.96 ml 0.1M phosphate buffer, pH 8.0; 20 µl microsomes fraction (1ml=1g fresh liver); and 10 µl, 10 Mm NADPH. The increase in fluorescence due to the formation of resorufin was recorded for 3 min and then 10 µl of 15 µM resorufin standard solution was added to record further increase in fluorescence. The protein content in microsomes fraction was determined by the method of Lowry et al. (1951) and EROD activity was calculated as pmol resorufin formed min^{-1} mg^{-1} protein.

RESULTS AND DISCUSSION

Adult sheim of more than one year of age were obtained from the hatchery and acclimatize in the laboratory before EROD assay in liver microsomes. Only adult fish were used in order to avoid variations in EROD activity reported in mature and juvenile fish in several species (Forlin, 1980; Koivusaari et al., 1981). The weights of the fish ranged around 300g and the basal activity obtained in control fish liver microsomes was 12.93±3.03 pmol min^{-1} mg^{-1} protein that was increased to 18.25± 5.25 pmol min^{-1} mg^{-1} protein by maintaining the fish in the laboratory as observed on day-4 (Table 1).

The optimum pH for EROD activity in sheim liver microsomes was found to be 8.0. Activity reduced marginally at pH 7.8 and drastically at pH 8.2. No activity was detected at pH 7.6, 7.4 or 7.2. Activity was also found to be NADPH-dependent, and omission of NADPH from the medium resulted in the loss of activity. The assay was run at room temperature, i.e., 25°C. The microsomes from sheim liver showed a characteristic CO spectrum for cytochrome P450 with absorption maxima at 450 nm.

The average EROD activity in sheim from the hatchery was more than three fold less than the activity obtained in fish caught in the Arabian Gulf in 1992 during the Mt. Mitchel cruise (Beyer and Goksoyr, 1993). The higher activity obtained in the field samples may be attributed to the exposure to a variety of pollutants present in the Gulf water following the 1990 Gulf war. An increase in EROD activity in fish liver with proximity to the area most affected by the 1991 oil spill was observed. EROD activity determination is a catalytic method of cytochrome P450 1A1 analysis. Among the biomarkers best described are monoxygenase in the cytochrome P450 system and especially in the 1A subfamily, in which P450 1A1 is the single known member in fish. Other studies have also shown induction in cytochrome P450 1A1 in response to exposure to petroleum products and PAHs (Payne and Penrose, 1975; Klienow et al., 1987; Goksoyr and Forlin, 1992).

BaP was used as a representative test chemical of PAHs for induction of cytochrome P450 1A1 in sheim. In general EROD activity in BaP exposed fish liver microsome was increased (Table 1). The lowest concentration tested was

Table 1. EROD induction on exposure to varying concentrations of BaP

Treatment (mg kg^{-1} wt. of fish)	EROD* Day 2	Fold Increase	EROD* Day 4	Fold Increase
0	12.93±3.03	1.0	18.25± 5.25	1.0
0.125	24.60±6.51	1.9	26.78±11.48	1.5
0.25	21.05±5.89	1.6	29.62± 9.28	1.6
0.50	20.07±10.43	1.6	36.76± 2.18	2.0
1.00	46.80±34.50	3.6	45.20± 3.51	2.5
2.50	56.65±22.63	4.4	63.44±21.35	3.5
5.00	ND	ND	725.10±337.5	33.7
10.0	ND	ND	1080.30±302.8	59.2
15.0	89.30±21.54	6.9	879.20±452.0	48.2
25.0	139.80±25.87	10.8	287.10±200.2	15.7

*pmol resorufin min^{-1} mg^{-1} protein

0.125 mg BaP kg^{-1} of sheim, which represents a tissue burden of 0.125 ppm . The weight of the fish used was around 300 g, and normally, fish are fed at a rate of 3% of their body weight, which suggests a requirement of 9 g of feed for a 300-g fish. Thus, by distributing the dose to feed required the levels come to 14 μg BaP g^{-1} food. This dose is close to the range of PAH concentrations that may be found in prey or in sediment that is ingested during feeding at heavily contaminated sites (Hugget et al., 1988; Bender and Hugget, 1987). Van Veld et al. (1997) used 10 μ g BaP g^{-1} food and fed the fish at 3% body weight with an amended floating aquarium diet for around 20 d to register induction in cytochrome P450 1A1.

In our study, a single exposure was given and the liver cytochrome P450 1A1 was determined fourth day after exposure. Therefore, a range of exposure concentrations were selected to establish the dose response relationship. The induction, as observed on the fourth day, at the lowest exposure concentration was 1.47 fold the basal levels, which was, however, statistically insignificant because the standard deviation of the two sets overlapped. Further increase in the dose caused an increase in EROD activity and a two fold induction was observed at a 0.5-mg kg^{-1} dose, which increased to 3.8-fold at 2.5 mg kg^{-1}. However, treatment dosages of 5 mg kg^{-1} up to 25 mg kg^{-1} resulted in a dramatic increase in EROD activity. At 5 mg kg^{-1} the activation was 33- fold, and it increased to 59-fold at 10 mg kg^{-1} doses. A further increase in dose to 15 mg kg^{-1} resulted in a decline in the activation to 48-fold the basal level. This decline was more visible at a 25 mg kg^{-1} dose when fold activation of only 15 times the basal level was obtained.

The induction in the EROD activity varied with exposure dosage, and at low doses induction was of a lower magnitude examined at either day-2 or day-4 of exposure. At higher doses significant increase in EROD activity was obtained even at day-2 of exposure. Therefore, induction and recovery of EROD in fish was followed up to 32 days after a single exposure to a 25-mg kg^{-1} dose. The induction in EROD was maximized on the fourth day and then started declining (Fig. 1). On

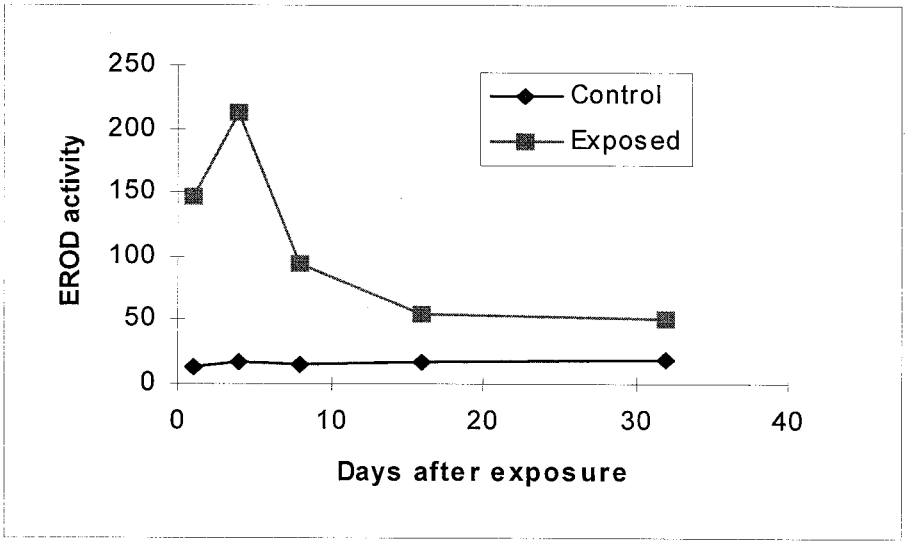

Figure 1. Induction and recovery in EROD with time after single exposure.

the eight day, the activity declined to one-half the level of the fourth day. At 16 d, there was a further drop in the level of enzyme activity. The level obtained at 16 d persisted up to 32 d. This level was around three fold higher than the level of enzyme activity in control fish. The data suggest an induction after exposure to BaP which initially increased, but subsequently, the induced enzyme slowly tended to return to the basal level since the exposure was not continuous.

The data with sheim showed a dose-response relationship for EROD induction over wide ranges of concentrations of BaP, suggesting that this induction might be used as a dosimeter in diverse varieties as observed in several other studies (Haasch et al., 1992). However, suppression of induction at extreme doses also needs attention to avoid misinterpretation. It may be kept in the mind that even at the highest dose a substantial increase in EROD was evident though the increase was little less than at lower than highest dose of treatment. In no case it was less than the basal activity obtained in unexposed fish. The data on the inducibility of EROD in sheim paves the way for its application as a sensitive biomarker in the monitoring of the pollution of the coastal waters in the Arabian Gulf region.

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